# TECHNICAL MANUSCRIPT 155

# RICKETTSIAL GROWTH IN SUSPENSION CELL CULTURES IN SERUM-FREE AND DEFINED MEDIA

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# RICKETTSIAL GROWTH IN SUSPENSION CELL CULTURES IN SERUM-FREE AND DEFINED MEDIA

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## ABSTRACT

Rickettsia typhi and Rickettsia rickettsii were propagated in cultures of cat kidney and L cell lines that were grown in a serum-free suspension system. One growth medium (LAH) consisted of lact-albumin hydrolyzate and glutamine as nitrogen sources, glucose and pyruvate as carbon sources, vitamins, salts, and additives such as Methocel, insulin, and phenol red. A second and third medium consisted of essential amino acids (EAA) with or without nonessential amino acids (NEAA), respectively as nitrogen sources, salts, carbon sources, vitamins, and the additives, Methocel, insulin and phenol red. LAH supplemented with 10 per cent calf serum was used as a control.

The two cell lines were carried as stock cultures in serum-free suspensions at least a month prior to the experiments. The studies were conducted with 100-ml serum bottles (30 ml medium per bottle) and were agitated on a New Brunswick shaker at 130 rpm at 35°C. A maximal yield of $10^{6.3}$ yolk sac (YS) $LD_{50}$ of R. typhi occurred at 7 days in cat kidney cells grown in EAA; titers were slightly less in LAH and considerably less in NEAA cell cultures.

In contrast, maximal yields of $10^{5.8}$ $YSLD_{50}$ of R. rickettsii occurred at 4 days in L cells grown in NEAA; slightly lower titers were found in EAA. In LAH only minimal amounts of rickettsiae were found. Titers obtained with cultures infected with R. typhi suggested cyclic rickettsial growth.

These data, obtained in serum-free and chemically defined media, demonstrate the importance of nutritional environments for maximum survival, penetration, and proliferation of rickettsial species and suggest a new approach to studying rickettsial metabolism.

## I. INTRODUCTION

Studies that define the nutritional requirements for rickettsial multiplication in animal cell cultures have received increasing attention during the last two decades. A limiting factor in these studies has been the need for a serum for the growth of animal cell cultures. A system in which experiments could be conducted with serum-free media or with chemically defined media would be desirable. Several such investigations have been reported[1,2,3] at meetings and in the literature in recent years. This paper presents the results of preliminary studies on the growth of Rickettsia rickettsii and Rickettsia typhi in suspended cell cultures employing serum-free and chemically defined media.

## II. MATERIALS AND METHODS

The media employed were as follows: (a) A serum-free lactalbumin hydrolyzate medium designated as LAH (Table I) which contained lactalbumin hydrolyzate and glutamine as nitrogen sources, glucose and pyruvate as carbon sources, vitamins, salts, plus such additives as Methocel and phenol red; (b) a defined medium designated as EAA (Table II), which contained 13 essential amino acids, carbon sources, salts, vitamins and Methocel and phenol red; and (c) the defined medium plus six amino acids found nonessential for cell growth, and hereafter referred to as NEAA. In studies with R. typhi, a fourth type of medium, LAH supplemented with 10 per cent calf serum, was employed. The medium with serum was added to serum-free cat kidney cells grown just prior to infection. This combination served as an initial positive control for the growth of rickettsiae.

Animal cell cultures, suspended in 30 ml of media contained in 100-ml serum bottles were incubated at 34°C in a New Brunswick gyrotory shaker rotating at 130 rpm. Both cat kidney and L cell lines were derived from stocks maintained in either the LAH medium or the defined medium for at least a month before infection with rickettsiae.

The Bitterroot strain of R. rickettsii and the Wilmington strain of R. typhi (R. mooseri) were prepared as inocula from infected yolk sacs of embryonated eggs by a modified, molar potassium chloride method of Ormsbee.[4] After the final centrifugation, rickettsiae were suspended in Bovarnick's[5] sucrose-phosphate-glutamate containing 0.6 per cent bovine albumin (Armour's Fraction V) and stored at -65°C until the mixtures were thawed and employed as inocula.

TABLE I. LACTALBUMIN HYDROLYZATE MEDIUM FOR SUSPENDED CELL GROWTH

| Component | Concentration, mg/l | Component | Concentration, mg/l |
|---|---|---|---|
| **Nitrogen Sources:** | | **Salts:** | |
| Lactalbumin hydrolyzate | 2500 | NaCl | 7400 |
| L-Glutamine | 300 | KCl | 400 |
| | | $NaH_2PO_4 \cdot H_2O$ | 100 |
| **Vitamins:** | | $NaHCO_3$ | 300 |
| | | $CaCl_2 \cdot 2H_2O$ | 265 |
| D-biotin | 1.0 | $MgCl_2 \cdot 6H_2O$ | 275 |
| Choline·Cl | 1.0 | | |
| Folic Acid | 1.0 | **Carbon Sources:** | |
| Niacinamide | 1.0 | | |
| Ca pantothenate | 2.0 | Glucose | 1000 |
| Pyridoxal·HCl | 1.0 | Sodium pyruvate | 110 |
| Thiamine·HCl | 1.0 | | |
| i-inositol | 1.0 | **Antibiotics, etc.:** | |
| Riboflavin | 0.1 | | |
| $B_{12}$ | 0.002 | Methocel 15 cps | 1000 |
| | | Phenol red | 10 mg/l |

TABLE II. CHEMICALLY DEFINED MEDIUM FOR SUSPENDED CELL GROWTH

| Component | Concentration, mg/l | Component | Concentration, mg/l |
|---|---|---|---|
| **Amino Acids (essential):** | | **Salts:** | |
| L-Arginine·HCl | 100 | NaCl | 7400 |
| L-Cysteine·HCl | 75 | KCl | 400 |
| L-Histidine·HCl | 60 | $NaH_2PO_4 \cdot H_2O$ | 100 |
| L-Isoleucine | 150 | $NaHCO_3$ | 500 |
| L-Leucine | 300 | $CaCl_2 \cdot 2H_2O$ | 265 |
| L-Lysine | 300 | $MgCl_2 \cdot 6H_2O$ | 275 |
| L-Methionine | 60 | | |
| L-Phenylalanine | 120 | **Carbon Sources:** | |
| L-Threonine | 135 | | |
| L-Tryptophan | 60 | Glucose | 1000 |
| L-Tyrosine | 120 | Sodium pyruvate | 110 |
| L-Valine | 150 | | |
| L-Glutamine | 450 | **Vitamins:** | |
| **Amino Acids (nonessential):** | | D-biotin | 1.0 |
| | | Choline·Cl | 1.0 |
| Glycine | 60 | Folic Acid | 1.0 |
| L-Alanine | 120 | Niacinamide | 1.0 |
| L-Serine | 150 | Ca pantothenate | 2.0 |
| L-Cystine·HCl | 75 | Pyridoxal·HCl | 1.0 |
| L-Aspartic Acid | 270 | Thiamine·HCl | 1.0 |
| L-Glutamic Acid | 315 | i-inositol | 1.0 |
| L-Proline | 115 | Riboflavin | 0.1 |
| | | $B_{12}$ | 0.002 |
| **Antibiotics, etc.:** | | | |
| Methocel 15 cps | 1000 | | |
| Phenol red | 10 mg/l | | |

Cell cultures with populations from $5 \times 10^5$ to $1.5 \times 10^6$ cells per ml were inoculated with these partially purified rickettsiae. The mixture was incubated for 45 minutes on the rotary shaker-incubator and centrifuged to sediment the cells. Supernatant medium and unadsorbed rickettsiae were removed by decanting, and the remaining cells resuspended in fresh medium. These resuspended cells were then sampled for a rickettsial assay and a cell count. The cultures were separated from spent medium by centrifuging every other day and resuspended in fresh medium. Additional culture samples were obtained daily, diluted 1:1 with normal calf serum, sealed in glass ampoules, and stored at -65°C until assayed for rickettsial titer in embryonated eggs. Viable tissue cell counts were made using the 0.5 per cent trypan blue method.

## III. RESULTS AND DISCUSSION

To assay rickettsial infectivity, tenfold dilutions of each sample were inoculated into the yolk sac of 7-day embryonated eggs. After incubating the eggs at 35°C in a commercial incubator for 11 days, embryo deaths were determined daily by candling. Embryo deaths occurring in the typical pattern for rickettsial infections were employed to calculate the $LD_{50}$ titer per ml by the method of Reed and Muench.

The stabilities of rickettsial infectivity at 34°C in a shaker-incubator were determined first in LAH and defined media without tissue cells. Ten per cent skimmed milk was included also for comparison. R. typhi and R. rickettsii showed similar losses of infectivity (Figure 1). Each lost one or more logarithms of infectivity after four hours at 34°C. Other data indicated little or no infectivity remained after 24 hours of incubation. These findings suggested that rickettsial infectivity found after 24 hours must have been a result of cell-rickettsial association.

When cells of the cat kidney line were infected with R. typhi (Figure 2), cultures grown either in LAH or LAH plus 10 per cent calf serum appeared to produce cyclic yields of rickettsiae. The highest yields of rickettsiae were about $10^8$ $ffLD_{50}$ per ml and occurred in the LAH medium on the 4th, 14th, and 20th day after inoculation, as shown by the open circles in the figure. The highest yield found for the cells grown in the medium with serum was $10^{6.5}$ infectious units and occurred on the ninth day of the infection. Neither rickettsial yields nor the cell proliferation appeared to be enhanced by the addition of 10 per cent calf serum to the LAH.

The number of cat kidney cells remained static for the first six days of rickettsial infection, then, a precipitous drop in the number occurred on the Day 7. This was followed by a slow decline in cell numbers until complete cell lysis occurred on Day 15 in the medium with serum, or on Day 20

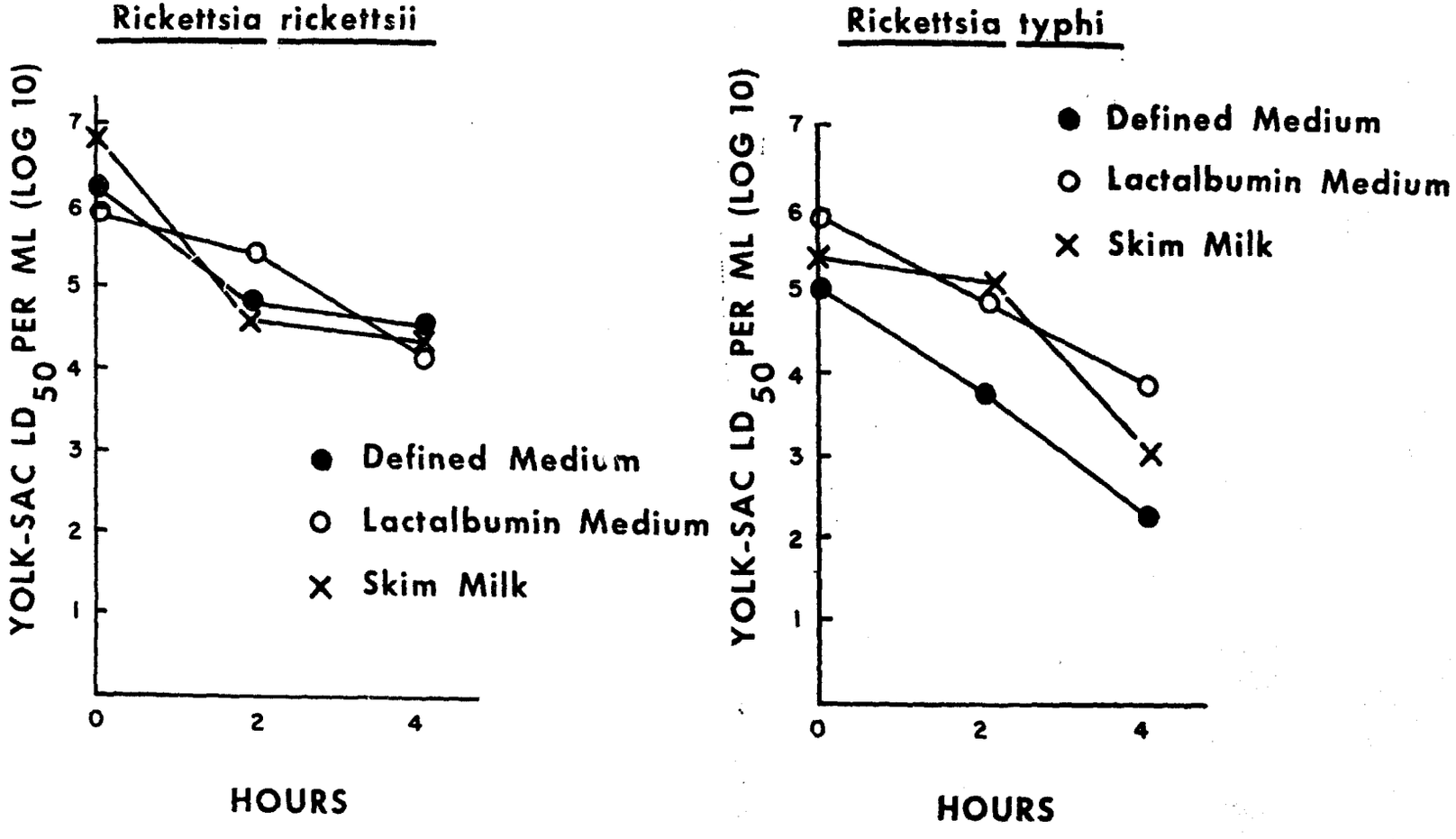


Figure 1. Stability of Rickettsia in Cell-Free Tissue Culture Media in a Gyrotory Shaker at 34°C.

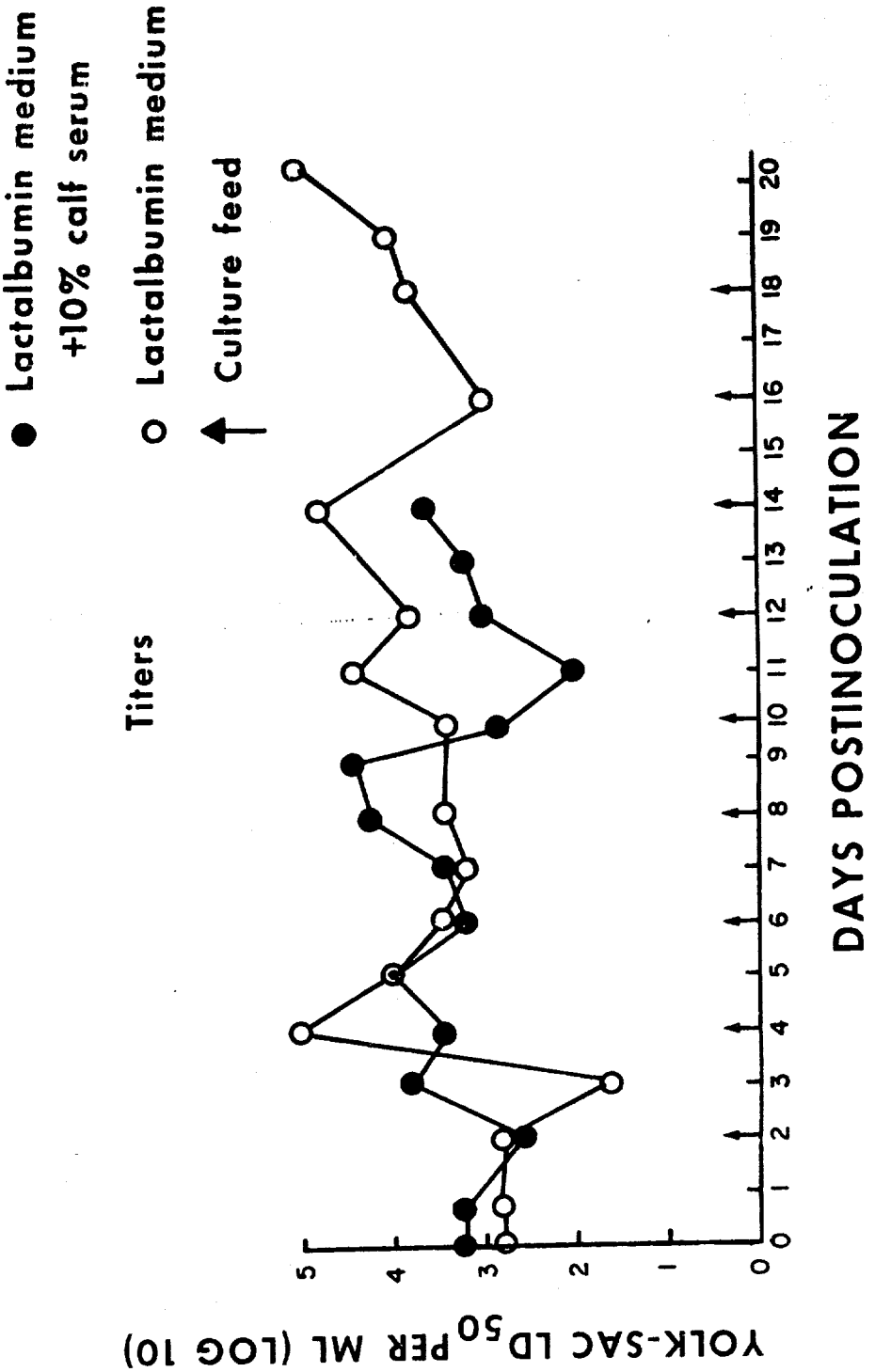


Figure 2. Growth of R. typhi in Cat Kidney Cell Line Suspension Culture.

in the LAH medium. Increases in the rates of cell lysis were generally associated with increased rickettsial yields.

When growth curves of R. typhi were determined in cultures of the cat kidney line propagated in EAA and NEAA media (Figure 3), the tissue cells grown in EAA yielded greater amounts of rickettsiae than did those cells grown in the same medium supplemented with nonessential amino acids. This is shown by the closed circles in the figure, which depict maximal rickettsial yields of $10^{8.8}$ $YSLD_{50}$ per ml in the defined medium, compared to $10^{8.2}$ $YSLD_{50}$ per ml for cells in the defined plus NEAA medium (open circles). A culture life of 8 days was not sufficient to confirm the cyclic yields of rickettsiae observed in the previous experiment. However, a comparison of the rickettsial yields with the number of cells in cultures again suggested that maximal infectivity was closely associated with cell lysis.

With R. rickettsii in L cell cultures the beneficial effect of incorporating the nonessential acids was observed (Figure 4). Cells infected with spotted fever rickettsiae and grown in NEAA produced a maximal yield of $10^{8.3}$ $YSLD_{50}$ per ml on the sixth day after infection, shown as the solid circles in the figure. By comparison, cells in the defined EAA medium produced only $10^{5.5}$ $YSLD_{50}$ per ml. In marked contrast, infected cells suspended in LAH medium grew well but failed to support proliferation of the rickettsiae.

In summary, a new approach to studying rickettsial metabolism has been demonstrated in cells propagated in serum-free and chemically defined environments. Preliminary data have suggested rather striking differences among media and rickettsial species for maximal survival, penetration, and proliferation.

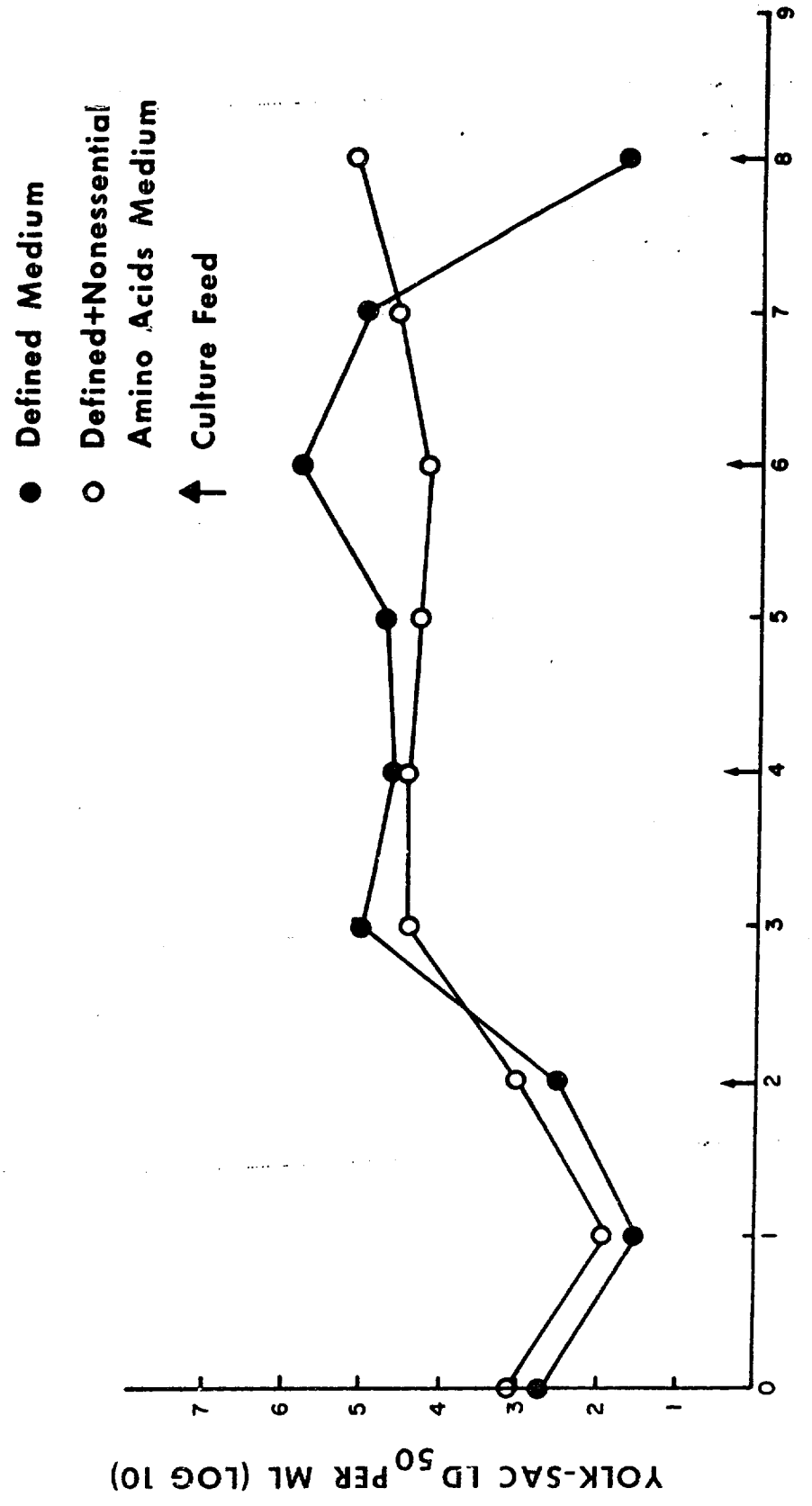


Figure 3. Growth of R. typhi in Cat Kidney Cells.

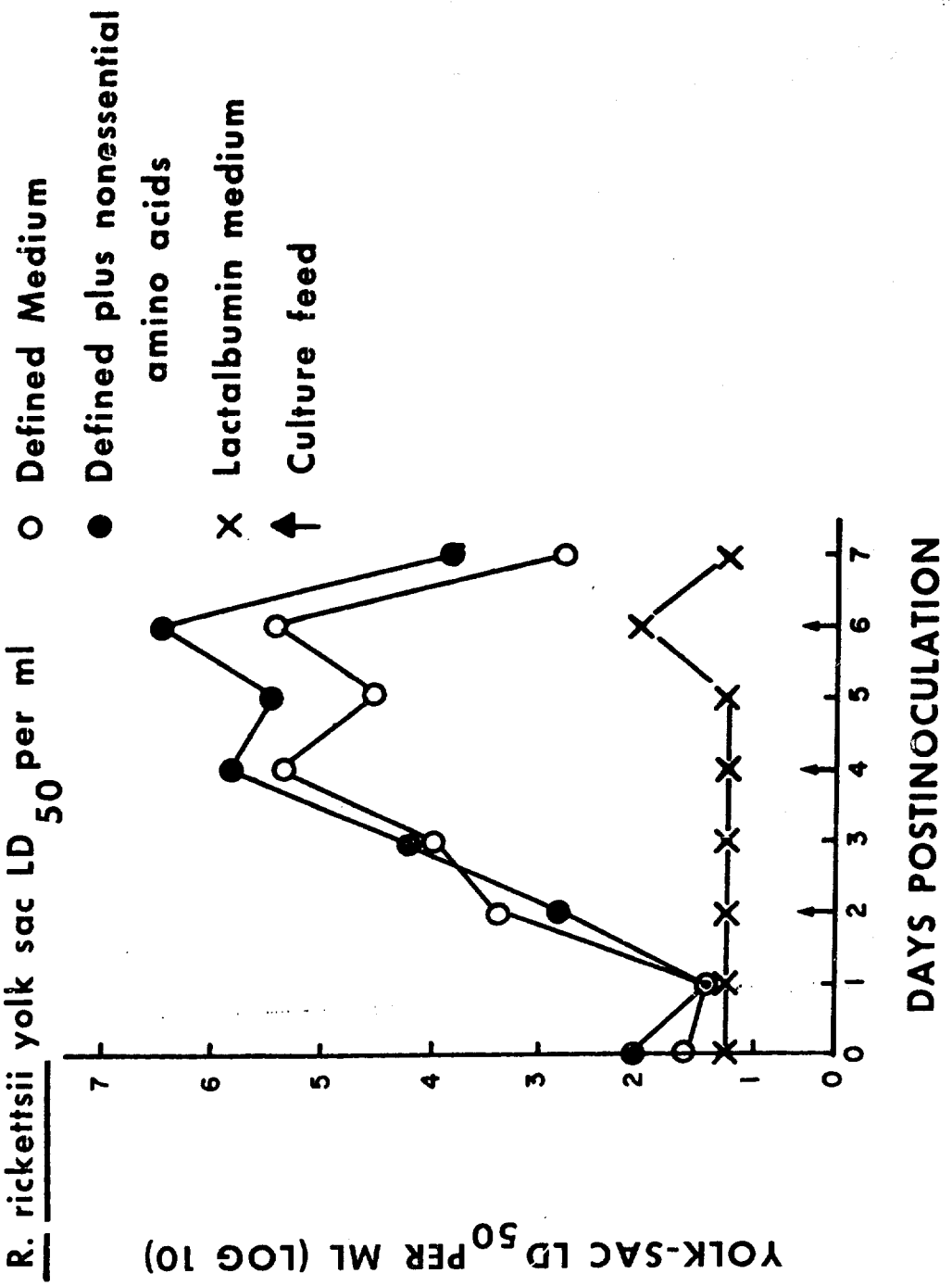


Figure 4. Growth of R. rickettsii in L Cell Suspension Culture.

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